VETERINARY SCIENCE RESEARCH JOURNAL
olume 7 | Issue 1 | April, 2016 | 31-38

DOI : 10.15740/HAS/VSRJ/7.1/31-38
Visit us - *www.researchjournal.co.in*
e ISSN-2230-942X

RESEARCH ARTICLE

Effect of pineal proteins/peptides of 10-20 kDa on immunomodulation in guineapigs for brucella abortus strain 19 vaccine

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Abstract : There existence of bilateral interactions between pineal gland and immune system. By this bidirectional interaction, pineal gland influences immune functions and immune signals affect pineal function. Microbial pathogens detected by immune system will produce an acute activation of immunocompetent cells. The activated immunocompetent cells modulate the pineal melatonin response, which inturn, will influence the specific immune response along with other melatonin sensitive neuroendocrine mechanisms.In this study the bubaline pineal proteins/ peptides fraction with., 200µg/kg b.wt.of 10-20 kDa was studied for its time dependent immunopotentiation effect in guinea pigs for the th $2.6x10^9$cells of live low virulence culture of Brucella abortus strain 19 vaccine at 04:00 hours and 16:00 hours .Blood samples were collected by cardiac puncture from all guinea pigs, on day 0, 7 , 14 and 21 post inoculation of vaccine and serum was separated. The serum agglutination antibody for B.abortus was first confirmed by RBPT and its titres were measured by STAT. The serum agglutination antibody level was the parameter of immunopotentioation in humoral immunity. The bubalinr pineal 10-20 kDa proteins/ peptides injected at 04:00 hour, significantly ($P<0.01$) increased the serum antibody levels (STAT) on day 14 and 21 as compared to vaccinated control by 586 66 and 1386.67 I.U./ml and its immunopotentiation efficiency by 122 and 144 per cent, respectively. By this study it is concluded that Injection of bubaline pineal proteins/peptides can modulate the immunity and also injection at 04:00 hours increased the immunopotentiation than at 16:00 hours thereby showing the chronobiotic effect.

Key words : : Bubaline pineal proteins/peptides, 10-20 kDa proteins/peptides, Brucella abortus strain 19 vaccine, Chronobiotic, Immunomodulation,Immunopotentiation

How to cite this paper : Ramasamy, M., Srivastava, R.S., Balagangathara thlagar, M. and Jayanthi, D. (2016). Effect of pineal proteins/peptides of 10–20 kDa on immunomodulation in guineapigs for brucella abortus strain 19 vaccine. *Vet. Sci. Res. J.*, **7**(1) : 31-38.

Paper History : Received : 02.02.2016; Revised : 25.02.2016; Accepted : 16.03.2016

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INTRODUCTION

The mammalian pineal gland secretes biologically active proteins, peptides and enzymes that have many physiological roles (Blask *et al.*, 1983). Numerous biochemical and behavioural functions of organisms are controlled

by the pineal gland through melatonin (MEL) and pineal protein secretions (Romijn,1978; Sejian, 2006 and Bharti and Srivastava, 2009). Pineal gland functions includeits ability to directly neutralize a number of toxic agents and stimulate antioxidative enzymes (Reiter *et al.,* 1995). During the last decade, a number of sometime contradictory reports appeared onthe role of the pineal gland in ageing (Armstrong and Redman, 1991; Treintini *et al.*, 1991; Reiter *et al.*, 1995; Reppert and Weaver, 1995; Anisimov, 1996 and Pierpaoli, 1998). Melatonin (*N*-acetyl-5-methoxy-triptamine) is the main pineal hormon synthesized from tryptophan predominantly at night time (Arendt, 1995). As age advanced, the nocturnal production of melatonin decreases in animals of various species as well as in human (Reiter, 1995; Touitou *et al.,* 1997 and Waldhauzer *et al.*,1998). Pinealectomized rats showed a reduced life span (Malm *et al.*, 1959 and Reiter *et al.*, 1999) whereas an administration of melatonin to mice, rats, fruit flies or planaria leads in some cases to the life extension (Pierpaoli *et al.*, 1991; Pierpaoli and Regelson, 1994; Oakin-Bendahan *et al.*, 1995; Anisimov *et al.*, 1997). The grafting of the pineal gland from young donors into the thymus of old mice or *in situ* into pinealectomized old mice prolonged the life span of the donors (Pierpaoli *et al.*, 1991 and Lesnikov and Pierpaoli, 1994). There is an evidence of production of some peptides by pineal gland (Yuwiler and Brammer, 1993 and Anisimov, 1998). It was shown that pineal polypeptide preparation Epithalamin® increases the life span of mice, rat and fruit flies and inhibits the development of spontaneous, induced by chemical and ionizing radiation and transplantable tumors in mice and rats (Anisimov *et al.*, 1982, 1989, 1994, 1997 and 1998). It is worth noting that clinical use of Epithalamin was shown as effective for treatment of ovarian disturbances and cancer incancer patients (Morozov and Khavinson, 1996) but rather restricted due todifficulties in the availability of calf pineal gland for it production. The synthesis of the pineal tetrapeptide Ala–Glu–Asp–Gly with high biologival activity (Khavinson and Morozov, 1999) gives a new opportunity for its implementation in clinical practice. It was recently shown that the pineal peptide increased the life span in two. The present study was conducted at izatnagar in Bareilly district (U.P). Adult female guinea pigs of Dunkin Hartley Strain, Obtained from Laboratory Animal Resource Section, IVRI, Izatnagar were used for this study.This study conduted to examine the immunopotentiation effect of bubaline pineal extract containing proteins and peptides of 10- 20 kDa agains Brucella abortus vaccine in gunea pigs.

RESEARCH METHODOLOGY

The gunea pigs. were provided with 40-50 g of feed/day and free access to drinking water. Brucella abortus strain-19 vaccine SAT antigen and anti brucella abortus serum obtained from Biological Product Division, IVRI, Izatnagar, was used for this study. It is a suspension of a pure smooth culture of Brucella abortus strain-99 in phenol saline. This SAT antigen was used for standard tube agglutination test in this study. B.melitensis B115 allergen is freeze dried purified skin test antigen of Brucella melitensis strain-115 cell in lyophilized state. This allergen was used for skin delayed type hypersensitivity test (SDTH) in this study.

Pineal glands from female buffalo (Bubalisbubalis) brain were collected from large animal abattoir, Mohanpur, Bareilly. The pineal was identified by its topographical location in brain (Getty, 1975). The extraction was carried out according to Bartsch *et al*. (2000). The homogenate of the glands was centrifuged at 7000 rpm for 1 hour at 4ºC (Remi, C-24, Remi India Ltd.). The supernatant was collected and filtered through 0.22 µm filter (Miller-GV, 0.22 µm, Millipore Mosheim, France).

The supernatant was sequentially passed through ultrafiltration membranes as described by Bartsch *et al*. (2000). The pineal proteins/peptides fraction with different molecular weights were separated using centrisart centrifugal ultrafilters having cut off at 100 kDa and 20kDa (Centrisart 13269 and 13249, Sartorius AG, Gottingen, Germany. The protein concentration of pineal fractions 10-20 kDa were estimated using Lowry's Method of protein quantification of BSA as standard and measuring absorbance at 550 nm in Spectrophotometer.

Employing denaturing polyacrylamide gel (20%) electrophoresis (PAGE) as per method of Laemmli (1970). Adult female guinea pigs of Dunkin Hartley strain weighing 500 to 600 gram each were used as experimental animals.

Rose bengal plate test (RBPT) was performed in sera as per WHO recommendation made by Alton *et al*. (1975) with Brucella abortus serum agglutination test (SAT) antigen containing a suspension of smooth culture of

Brucella abortus strain-99 in phenol saline. The guinea pigs whose sera exhibited ≥30 international serum agglutination units per ml (I.U/ml) or titre ≥15 was considered as positive for brucella infection (as recommended by Bercovich *et al.,* 1995).

The proteins/ peptides fraction with., 200µg/kg b.wt.of 10-20 kDa was further studied for its time dependent immunopotentiation effect.

Adult female guinea pigs were randomly divided into six groups, each consisting of 6 guinea pigs. Two group (Gr. PV4 and Gr.PV15) were experimental ones for pineal proteins/peptides of 10-20 kDa, another two group (Gr. VC4 and Gr.VC16) were control or vaccine and further two more groups (Gr. NC4 and Gr. NC16) were control for homogenizing fluid. Group PV4 and PV16 were injected at 04:00 and 16:00 hour with 200µg of pineal proteins/ peptides of 10-20 kDa per kg b.wt through subcutaneous (S/C) route daily for seven consecutive days. The animals in Gr. NC4 and NC16 were injected only with homogenizing fluid at 04:00 and 16:00 hours, respectively. On day 4 of the experiment, the guinea pigs in Gr.PV4 and VC4 were inoculated with $2.6x10^9$cells of live low virulence culture of Brucella abortus strain 19 vaccine at 04:00 hours and in Gr. PV16 and VC16 were inoculated with same dose of Brucella abortus strain-19 vaccine at 16:00 hours of the same day by intramuscular route on left hind leg as described by Thornton and Musket (1972).

Blood samples were collected by cardiac puncture from all guinea pigs, on day 0, 7 , 14 and 21 post inoculation of vaccine and serum was separated. The RBPT was done to confirm the Brucella infection as WHO recommendation by Alton *et al*. (1975) with Brucella abortus serum agglutination test (SAT) antigen.

The blood samples were collected before and day 7, 14, 21 post inoculation of vaccine from 04:00 hour groups and 16:00 hour groups at 04:00 and 16:00 hour, respectively. Serum was separated and serum agglutination antibody for B.abortus was first confirmed by RBPT and its titres were measured by STAT. The serum agglutination antibody level was the parameter of immunopotentioation in humoral immunity.

RESULTS AND DISCUSSION

The serum samples of Gr.NC4 and NC16 were negative to RBPT on day 0, 7, 14 and 21 post inoculation. The serum samples of Gr.VC4, VC16, PV4 and PV16 were negative to RBPT on day before inoculation of vaccine but on day 7, 14 and 21 post inoculation of vaccine they were positive to RBPT test. The Serum samples of Gr.NC4 and NC16 were showed negative to STAT on day 0,7,14 and 21 post inoculation of vaccine. The serum samples of Gr.VC4, VC16, PV4, and PV16 were negative to STAT on day before inoculation of vaccine but on day 7, 14 and 21 post inoculation of vaccine the were positive to STAT.

In Gr.NC4, the serum samples were negative for STAT on day 7, 14 and 21. In Gr. VC4, the serum antibody level on day 7, 14 and were 266.67± 33.73, 480.00 ± 71.55

And 960.00±143.00 I.U./ml, respectively. As the days increased the serum antibody levels also significantly ($P<0.01$) increased. On day 14, the serum antibody level was non-significantly ($P<0.01$) higher on day 21. In Gr.Pv4, the serum antibody level on day 7, 14 and 21 were 533.33±67.46, 1066.66±134.92 and 2346.67±213.33 I.U./ml, repectively. As the days of experiment increased the serum antibody level also significantly ($P<0.01$) increased. The antibody level on day 14 was significantly ($P<0.01$) higher than that on day 7 and furthersignificantly ($P<0.01$) higher

Table 1: ANOVA of data on serum agglutination antibody level in different groups of guinea pigs injected with 10-20 kDa pineal proteins/peptides and B.abortus strain-19 vaccine

Source	d.f	Serum agglutination antibody level
Between groups	3	2194063.00**
Between periods	2	10779730.00**
Groups X periods	6	613688.80**
Error 1	17	246091.70
Error 2	34	109274.10

on day 21. The ANOVA on data of antibody levels revealed that the antibody levels differed significantly (P<0.01) between VC4 and PV4 groups. The interaction between group and period showed that increase in antibody level on day 14 and 21 was significant (P<0,01). The serum antibody level on day 7 was non-significantly higher and both on day 14 and 21 were significantly (P<0.01) higher in Gr. PV4 than that in Gr. VC4.

In Gr PV4, 200 Ng/kg b. wt dose of 10-20 kDa proteins/peptides injected at 04:00 hour, non-significantly increased the serum antibody level (STAT) on day 7 as compared to Gr VC4, by 266.66 I.U./m1 and its immunopotentiation efficiency by 99.0 per cent. This dose significantly (P<0.01) increased the serum antibody levels (STAT) on day 14 and 21 as compared to vaccinated control by 586 66 and 1386.67 I.U./ml and its immunopotentiation efficiency by 122 and 144 per cent, respectively.

Immunopotentiation by injection at 16:00 hour :

In Gr.NC16, the serum samples were negative for antibody levels (STAT) on day 7, 14 and 21. In Gr. VC16, the serum antibody levels (STAT) on day 7, 14 and 21 post inoculation of vaccine were 186 66±26.66, 373.33±53.33 and 693.33±128.44 I.U./ml, respectively. As the days increased the serum antibody level increased non-significantly. In Gr. PV16, the serum antibody levels (STAT) on day 7, 14 and 21 were 373.33±53.33, 853.33±134.92 and 1600.00±320.00 I.U./ml, respectively. As the days of experiment increased the serum antibody level also significantly (P<0.01) increased. The antibody level (STAT) on day 14 was significantly (P<0.01) higher than that on day 7 and further increased significantly (P<0.01) on day 21. The interaction between groups and periods showed that increase in antibody level on day 14 and 21 was significant (P<0.01) The serum antibody levels (STAT) on day 7 and 14 did not vary significantly between Gr. PV16) and VC16. However, these values were non-significantly higher in Gr. PV16 than those in Gr VC16. The serum antibody level (STAT) on day 21 in Gr. PV16 was significantly (P<0.01) higher than that in Gr. VC16.

Gr. PV16, 200 Ng/kg b.wt dose of 10-20 kDa proteins/peptides injected at 16:00 hour. non-significantly increased the serum antibody level (STAT) on day 7 and 14 as compared to vaccinated control by 186.67 and 480.00 I.U./ml and its immunopotentiation efficiency by 100 and 128 per cent, respectively. This dose significantly (P<0.01) increased the serum antibody level (STAT) on day 21 as compared to vaccinated control by 906,67 and its immunopotentiation efficiency by 130 per cent.

Comparative time dependent effects :

In the present study the administration of proteins/peptides at 4 00 hour showed non-significantly higher immunopotentiated serum antibody level than that at 16:00 hour on day 7 (266.66 Vs. 186.67 I.U/m1) and on day 14 (586,66 Vs. 480.00 I.U/m1) and also showed significantly (P<0.01) higher immunopotentiated antibody level on day 21 (1386.67 Vs 906.67 1.11/m1). This could be due to the active participation of immune and pineal function to improve the immune status. Similarly, Guerrero and Reiter (1992) suggested a direct link between pineal gland and immune system supported by considerable amount of accumulated experimental evidences. They hypothesised the

Table 2: Effects of injection of 10-20 kDa pineal proteins/peptides at 04:00 Vs 16:00 hour on serum agglutination antibody levels (I.U./ml) in guinea pigs inoculated with B.abortus S-19 vaccine

Group no.	Groups	Hours of inoculation	Number of animals	Days post inoculation		
				7	14	21
PV4	10-20kDa, 200µg/kg +B.abortus S-19	04:00	6	533.33 ± 67.46^{A}	$1066.66\pm134.92^{B,a}$	$2346.67\pm213.33^{C,a}$
PV16	10-20 kDa, 200 µg/kg + B.abortus S-19	16:00	6	373.33 ± 53.33^{A}	$853.33\pm134.92^{B,ab}$	$1600.00\pm320.00^{B,b}$
VC4	B.abortus S-19	04:00	6	266.67 ± 33.73^{A}	$480.00\pm71.55^{AB,b}$	$960.00\pm143.10^{B,c}$
VC16	B.abortus S-19	16:00	6	186.66 ± 26.66^{A}	$373.33\pm53.33^{A,b}$	$693.33\pm128.44^{A,c}$
NC4	Control (Homogenizing fluid)	04:00	6	0	0	0
NC16	NC16 Control (Homogenizing fluid)	16:00	6	0	0	0

Means bearing different superscripts with A,B,C differ significantly at P<0.01 between days within group

existence of bilateral interactions between pineal gland and immune system. By this bidirectional interaction, pineal gland influences immune functions and immune signals affect pineal function. Maestroni (1992) considered the pineal-immune network as a sensory organ. He viewed the pineal gland as a sophisticated immuno-neuro-endocrine network. Microbial pathogens detected by immune system will produce an acute activation of immunocompetent cells. The activated immunocompetent cells modulate the pineal melatonin response, which inturn, will influence the specific immune response along with other melatonin sensitive neuroendocrine mechanisms. Blask *et at*. (1983) concluded that some of the effects of pineal gland may be due to pineal peptide. During postnatal life of mice either surgical pinealectomy (Csaba and Barath, 1975) or pharmacological inhibition of pineal gland function (Maestroni *et al*., 1987) caused involution of thymus, associated with a depression of cell mediated immune response and significantly reduced antibody production.

In this study it could be concluded that the pineal proteins/peptides may evoked antibody reponse and B.abortus strain-19 vaccine. Skwarlo-Sonta (2002) also concluded that both strategic (developmental, thus antigen independent) and emergency (evoked by antigenic activation of mature immune system) levels of interaction are present between pineal gland and immune system so that the immunity in this study was improved.

In this study vaccination and injection of immunopotentiating proteins/peptides injected at 16:00 hour. non-significantly increased the serum antibody level (STAT) on day 7 and 14 as compared to vaccinated control by 186.67 and 480.00 I.U./ml and its immunopotentiation efficiency by 100 and 128 per cent, respectively. This dose significantly ($P<0.01$) increased the serum antibody level (STAT) on day 21 as compared to vaccinated control by 906,67 and its immunopotentiation efficiency by 130 per cent. Cassone *et al.* (1993) concluded that the neurohormone, melatonin was not naturally present through out the day, but instead secreted only during the night on a circadian basis in all animals. The possible involvement of endogenous melatonin on humoral and T cell immune reactions, as well as on spleen and thymus cellularity in mice was reported by Maestroni *et al*. (1986) Maestroni *et al*. (1986) reported that endogenous melatonin modulated the antibody response and antagonized the immunosuppressive effect of corticosterone.

Anisimov *et al*. (1994) observed that in the morning, a single injection or a 5 days course of injection of low molecular weight pineal peptide was followed by an increase of the night level of Serotonin, N-acetyl serotonin and melatonin in the pineal gland of rats. But exposure to low molecular weight pineal peptide at evening 18:00 hours was followed by a decrease in the night peaks of Serotonin, N-acetyl serotonin and melatonin in the rat pineal gland.

In the present study the proteins/peptides along with vaccine injected at morning 04:00 hours increased the serum antibody level on day 14 and 21 were significantly ($P<0.01$).This could be due to the chronobiotic and immunomodulatory effect of bubaline pineal proteins/peptides. Similar chronobiotic and immunomodulatory effect of melatonin administration at 4:00 and 16:00 hours in male rats against Pasteurellamultocida broth vaccine was studied by Korde (1997) and found that melatonin augumented cell mediated as well as humoral immune response in rats inoculated at 4:00 hrs with Pasteurellamultocida broth vaccine + Melatonin as compared to control. He concluded that administration of Vaccine +melatonin at 4:00 hrs led to immunopotentiating effect and that at 16:00 hrs led to marginal immuno suppressive effect.

In the present study the effects of low molecular weight bubaline pineal proteins/peptides of 10-20 kDa injected at 04:00 hours increased the immunopotentiating effect by increased production of antibodies. Similarly, Vassilijev *et al.* (1994) also concluded that injection of low molecular weight peptides obtained from pineal gland of bovine, influences the pineal gland of rats by increasing the synthesis and secretion of melatonin and by its modulating influences on immune functions. Anisimov *et al*. (1994) concluded that mechanism of the biological effects of low molecular weight peptides of pineal gland which includes the enhancement of the night peak of melatonin in the pineal gland and serum, lowering the hypothalamic threshold of sensitivity to homeostatic feed back stimuli, the modulation of some T and B cell mediated immune functions.

In mice, injection of low-molecular weight protein extracts from the pineal gland showed an increased number of antibody forming cells generated in the spleen and an increased level of serum haemagglutinins in response to sheep red blood cells (Berlokrylov *et al.,* 1976 and Anisimov *et al.,* 1982). Vassilijev *et al.* (1994) observed an enhanced antibody production to, T-dependent as well as T-independent antigen by repeated injection of low molecular

weight peptide of pineal gland. A single injection of peptide given on the third day of primary immunization also enhanced antibody production and delayed type hypersensitivity reaction. Tandon (2002) showed that the bubaline pineal cell free peptide fraction with 5-100 kDa at the dose of 100 pg/kg potentiated the humoral and cell mediated immune responses in goat against the bovine serum albumin. So the present study concluded that bubaline pineal proteins/peptides of 10-20 kDa potentiated immunity by immunomodulation and also shown chronobiotic effect.

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